Analysis of Birt-Hogg-Dubé Syndrome

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ABSTRACT

Birt-Hogg-Dubé syndrome (BHD) is a rare autosomal dominant disorder characterized by skin lesions of the face and neck known as fibrofolliculomas; the disorder is associated with an increased risk of collapsed lungs, cysts of the lungs, and renal cancer. The Folliculin gene is located on chromosome 17p11.2 and codes for the corresponding Folliculin protein. This protein which is inactivated within BHD individuals is postulated to function as a tumor suppressor, and has been associated with various signal transduction pathways: mTOR signaling, Ras-Raf-MEK-Erk signaling, AMPK signaling, HIF signaling, JAK/STAT signaling, TGF-β, apoptosis and aspects of the cell cycle. The precise molecular function of Folliculin has yet to be elucidated, yet it has been implicated in these signaling systems. No treatment or cure for BHD exists, consequently, patients are treated for clinical manifestations of the syndrome and undergo regular pulmonary and renal screenings. Currently, The National Institute of Cancer is recruiting for a study to determine the potential efficacy of the mTOR inhibitor, everolimus, as a treatment for BHD associated renal cancer.

EPIDEMIOLOGY & CLINICAL MANIFESTATIONS

The epidemiology and the incidence of BHD syndrome is not well defined but it is thought to be under-diagnosed. However, the prevalence is estimated to be 1 in 200,000. BHD is not defined by particular symptoms and individuals vary in their clinical manifestations.1 When this syndrome is observed, it generally occurs after 20 years of age but may manifest as young as 7. Skin lesions are the most prevalent of symptoms occurring in approximately 85% of individuals with BHD.2 These fibrofolliculomas occur on the neck, face, scalp, earlobes, and less frequently in the oral mucosa. Moreover, individuals exhibit varying degrees of fibrofolliculomas severity ranging from a few lesions to several hundred. Unfortunately, around 80% of BHD patients develop pulmonary cysts in both lungs. The pulmonary cysts are typically asymptomatic yet approximately 30% of affected individuals may experience spontaneous pneumothorax. Additionally, 15-30% of patients with BHD syndrome develop renal neoplasms. The two most common tumor types found in these patients are malignant and include hybrid oncocytic tumor and chromophobe renal carcinoma.

Diagram/Biomarkers/Genetics (FLCN mutations)

We now know that Birt-Hogg-Dubé is caused by an inherited autosomal dominant mutation within the FLCN gene which has been mapped to chromosome 17p11.2.4 Haplotype analysis of 4 families with a combined total of 23 affected individuals using BHD linked markers indicated that the affected alleles were not the same, excluding common ancestry. Mutation analysis of the Birt-Hogg-Dubé gene identified germline mutations on exon 11 (c.1733delC and c.1733insC) in 3 of 4 familial cases and 2 of 4 sporadic cases. Within exon 11 a (C) 8 tract is considered to be particularly prone to mutation. The repeating cytosine mononucleotide tract mutation was found in 44% of unrelated patients.5 Interestingly, a study conducted on a large French family with 8 affected individuals carrying the c.1733delC mutation exhibited no renal tumors. This may have been due to reduced penetrance.4 A study conducted on 51 families indicated that 27 families exhibited deletion (c.1285delC) or duplication (c.1285dupC) of the C nucleotide in exon 11, indicating these regions are mutational hotspots.6,7 A diagnosis of BHD occurs through genetic testing. The test method performed is a bi-directional sequencing of the splice sites of exon 4-14 of the FLCN gene as well as the coding regions. If the sequencing analysis does not indicate a mutation, a defined array which uses comparative genomic hybridization (CGH) allows exon-level resolution to detect deletions or duplications of the exons of the FLCN gene.5

Figure 1: Image A is a Chest X-Ray exhibiting the left spontaneous pneumothorax of a patient with Birt-Hogg-Dubé. Image B is a CT scan indicating the presence of multiple right lung cysts in an individual afflicted with BHD.3

Figure 2: This figure is an antisense strand of the DNA sequence which has the altered mononucleotide (Cytosine) 8 insertion. The insertion germline mutation is believed to produce a truncated Folliculin protein.9
FOLLICULIN PROTEIN: TUMOR SUPPRESSOR

Though it is unclear what functional activity the Folluculin protein is responsible for, it clearly behaves like a tumor suppressor. To examine the location of Folluculin mRNA expression, studies were performed using fluorescent in situ hybridization on both normal and neoplastic human tissue. Using this detection strategy, FLCN mRNA expression was found in: the distal nephron of the kidney, type 1 pneumocytes of the lung, acinar cells of the pancreas and parotid gland, skin, stromal cells, breast and pancreatic epithelial ducts, neurons of the cerebrum, Purkinje cells of the cerebellum, lymphocytes and macrophages of the spleen and tonsils, heart (reduced expression), muscle (reduced expression), liver (reduced expression), epithelial strands of fibrofolliculomas (strongly expressed), and cutaneous lesions (strongly expressed). Supporting the tumor suppressor argument, FLCN mRNA was not expressed in the renal tumors of BHD patients. Typically, renal tumors of BHD patients have expressed germline mutations within the FLCN gene as well as somatic inactivation of the remaining FLCN allele either through loss of heterozygosity or somatic mutations.

A transgenic mouse model of BHD was created to further examine the role of the FLCN protein. These studies, inserted an in-frame β-galactosidase-neomycin-phosphotransferase II gene into the FLCN gene between exons 8 and 9. Phenotypically, these mice mimicked the loss of function mutations characteristic of BHD because the insertion generated a heterozygous loss of FLCN. Homozygous deletions of the FLCN protein have also been constructed in mice. Unfortunately, these mutant mice result in embryonic lethality before embryonic day 8.5. Further, FLCN+/− mice presented with normal kidneys, whereas heterozygous FLCN+/− mice demonstrated sporadic renal tubule hyperplasia with single cysts as well as multiocular polycystic kidneys. The FLCN−/− mice did not exhibit skin lesions or lung cysts.

To further examine the FLCN−/− tissue morphology, kidney sections of heterozygous mice were simultaneously stained for Lotus tetragonolobus agglutinin (LTA), a proximal tubule marker, and FLCN. The renal cysts identified in these sections expressed the marker for LTA; however the epithelial lining of the cysts in the cyst-associated tissues did not express FLCN. This observation suggests that the renal cysts originate from the proximal tubules due to the loss of FLCN expression.

Additional studies were conducted to determine if the loss of the FLCN protein in the transgenic mice induced abnormal cell proliferation and/or apoptosis during renal cyst development. These studies stained kidney tissue from FLCN+/+ and FLCN−/− mice for the proliferation marker Ki-67. Though no difference in the cellular proliferation was observed in the renal cysts, the epithelial cell lining of the cysts and the tissues surrounding the cysts showed a drastic increase in Ki-67 staining compared to the normal kidney. Finally, immuno histochemical staining of these same kidney tissues for the apoptosis marker caspase-3 showed that neither the FLCN+/+ nor FLCN−/− mice had apoptotic cells.

Clear cell renal carcinoma (CC-RCC) is a histological subtype of renal cell carcinoma found in BHD patients. To determine whether FLCN is an independent tumor suppressor, two different human RCC cells were analyzed. The analysis was performed in two human RCC cell lines. One cell line (786-0) is VHL negative, while the other cell line (ACHN) is VHL positive. VHL corresponds to a known tumor suppressor, the von Hippel-Lindau tumor suppressor gene. FLCN was down regulated in the VHL positive (ACHN) cell line and overexpressed in the VHL negative (786-0) cell line. However, neither cell line exhibited notable effects in growth during overexpression or down regulation of the FLCN protein. Additionally, these studies showed no FLCN dependent changes in the levels of HIF2α (VHL target gene). Taken together, these studies suggest that the VHL tumor suppression and the FLCN tumor suppression occurs through independent pathways. Finally, FLCN expression levels were shown to have a significant effect in the tumor growth of ACHN and 786-0 cells when injected subcutaneously into athymic nude mice. Further, when the FLCN gene was reintroduced into the 786-0 cells there was a drastic decrease in tumor growth. Moreover, FLCN knockdown in ACHN cells resulted in the formation of large tumors.

Figure 3: Comparison of Ki-67 positive cells in FLCN+/+ kidney tissue, FLCN−/− kidney tissue, and FLCN−/− cystic epithelia. The data is a mean percentage of Ki-67-positive cells/total cells per field.

FOLLICULIN INTERACTING PROTEIN 1 & FOLLICULIN INTERACTING PROTEIN 2

Two Folluculin interacting proteins, (FNIP1 and FNIP2) have been identified through co-immunoprecipitation with Folluculin. Using in vitro translated 35S translated FNIP1 and genetic fusions of GST and FLCN it was shown that FNIP1 binds to FLCN with a proper C terminus but is unable to bind to FLCN proteins lacking the normal C-terminus. Additional studies have implicated FNIP1 and FNIP2 in AMPK and mTOR pathways. Using additional in vitro binding assays, it
was determined that the full length of FNIP1 was required for maximum binding to FLCN. Additional work was performed using Human Embryonic Kidney 293 (HEK 293 cells) to determine if a binding complex of FLCN-FNIP1-AMPK could be demonstrated. These studies transfected HEK 293 cells with an expression vector (HA-FLCN) with or without a FLAG-FNIP1 expressing vector. Extracts derived from these transfections were first immunoprecipitated using anti-Flag antibody followed by a Western blot analysis of the precipitated complex. Importantly, all of the AMPK subunits co-immunoprecipitated with HA-FLCN in a manner that was dependent on FLAG-FNIP1, confirming that AMPK does not bind to FLCN alone but is dependent on the FLCN-FNIP1 complex.

An additional study conducted in 2015 attempted to elucidate the functional roles of FNIP1 and FNIP2 in renal cancers by using murine models. By crossing mice with lox-flanked FNIP1 alleles with CDH16-Cre transgenic mice the FNIP1 gene was deleted specifically in kidney tissue. No significant renal phenotypes were observed within the FNIP1 knock out mice except for the occasional development of small cysts. FNIP1 deficient mice did exhibit phenotypes similar to FLCN deficient mice in heart and skeletal tissue. FNIP1 knockout mice exhibited cardiac hypertrophy with increased mTORC1 activity and increased mitochondrial biogenesis in skeletal tissue. Crossbreeding of lox-flanked FNIP2 allele mice with CDH16-CRE transgenic mice deleted the FNIP2 gene. Similar to the phenotypic observations of FNIP1 knockout, loss of FNIP2, did not cause the mice to develop renal tumors.

Due to the sequence similarity of FNIP1 and FNIP2 as well as the common interaction with FLCN and AMPK, a functional redundancy between the two interacting proteins was postulated. To demonstrate this, studies showed that expression of either FNIP1 or FNIP2 in FNIP1/FNIP2 null mouse embryonic fibroblasts suppressed ATP production and peroxisome proliferator-activated receptor gamma activator 1-alpha (PPARCGC1α), a protein involved in mitochondrial biogenesis. Due to the functional redundancies it was hypothesized that FNIP1 and FNIP2 may have variable expression in differing organs, and the expression levels may determine the specific roles for the respective proteins. Using rtPCR to measure mRNA copy number in FNIP1 and FNIP2 wild type mouse tissue, it was observed that FNIP1 is primarily expressed in heart tissue, skeletal tissue, and bone marrow. Further, no significant difference was seen in expression levels between FNIP1 and FNIP2 in kidney tissue. Consequently, it is reasonable to believe that expression of one of the interacting proteins might compensate for the inactivation of the other. This, of course, suggests that double inactivation of FNIP1 and FNIP2 is necessary for development of BHD renal phenotypes. In fact, studies of mice, which are deficient in both FNIP1 and FNIP2, demonstrate that they develop enlarged renal cysts (Figure 4).

**FOLLCULIN AND THE MTOR SIGNAL TRANSDUCTION PATHWAY**

The serine/threonine kinase, mammalian target of rapamycin (mTOR), is an effector of cellular metabolism, proliferation, and growth, acting through direct and indirect phosphorylation of downstream targets. Dysregulation of mTOR has been implicated in human cancer. mTORC1 and mTORC2 are two distinct multiprotein complexes which contain mTOR. mTORC1 is a rapamycin sensitive complex which activates S6K1 and 4EBP1, as well as proteins involved in mRNA translation. Further, mTORC1 is activated by growth factors, energy signals, stress signals, and nutrients. mTORC2 is considered rapamycin resistant, and is involved in the regulation of the actin cytoskeleton. Notably, mTORC2 is activated by growth signals. Constitutive activation of mTOR is caused by activation of oncogenes or the loss of function of tumor suppressors. Overexpression of growth factors such as IGFR (insulin like growth factor receptor) and HER2 (human epidermal growth factor receptor 2) cause the activation of the mTOR pathway. The mTOR pathway is involved in the PI3K-AKT-mTOR axis. Consequently, mutations in PI3K, can constitutively activate mTOR and AKT pathways. mTOR activation increases the biogenesis of ribosomes providing the cell with the proteins necessary to proliferate and maintain a high growth rate.

In order to determine the role of Folliculin as a regulator of mTOR, heterozygous FLCN (FLCN+/−) mouse models were developed. FLCN+/− mice exhibited drastically increased levels of AKT protein in cancerous kidney tissue compared with normal kidney tissue. AKT levels were further increased at the mTORC2 phosphorylation site. Increased phosphorylation of the downstream effectors of AKT (p-GSK3, p-FOXO1, and p-FOXO3a) was also observed in cancerous renal tissue. Despite the increased level of total AKT protein, the mRNA levels of AKT were unchanged between cancerous and normal tissue, therefore FLCN may have a posttranscriptional or posttranslational modification role in the regulation of AKT. Increased mTOR phosphorylation on serine 2448 and serine 2481, which are indicative of mTORC1 and mTORC2 activation, respectively, were found in cancerous renal tissue but absent in normal kidney tissue. Furthermore a high level of AKT phosphorylation at serine 473 was indicative of mTORC2 activation. Rictor, which is a subunit and regulator of mTORC2, had increased levels in BHD+/- mice. It was postulated the primary activation of the AKT pathway is due to Rictor overexpression leading to activation of mTORC2.
Hasumi et al. had previously found that rapamycin partially blocked FLCN phosphorylation. Taken together, this information suggests that FLCN is involved in a negative feedback loop which suppresses the PI3K-AKT-mTOR signaling pathway (Figure 5). Western blotting and immunofluorescent staining of human BHD kidney tissue compared to normal tissue suggests there is an activation of the PI3K-AKT-mTOR pathway which is analogous to that found in the heterozygous FLCN (FLCN+/-) mouse models.

Furuya et al. analyzed the lungs of 11 patients with BHD to evaluate mTOR expression in pulmonary cysts. Immunostaining of the pulmonary tissue was done with rabbit polyclonal antibodies against FLCN, p-mTOR, p-S6, and mouse monoclonal antibodies against PCNA, TTF-1, and β-catenin. The cystic cells positively stained for p-S6, indicating activation of the mTOR pathway p-S6 is a downstream effector of mTOR. Conversely, in normal lung tissue, expression of p-mTOR and p-S6 was negative.

Hartman et al. determined contrasting data in regards to mTOR activity whilst attempting to determine the Folliculin mediated regulation of mTOR. To create transgenic mice with heterozygous loss of Folliculin an embryonic stem cell line was used. The embryonic stem cell line contained a β-galactosidase/neomycin cassette between exon 8 and 9 in the FLCN gene, which created a truncated FLCN protein. To determine whether mTOR was dysregulated in the FLCN-/- mice, the cystic tissue was stained with anti-phospho-S6 ribosomal protein. The oncocytic tissue stained negative for p-S6.

A 2008 study on Folliculin interacting proteins and Folliculin conducted by Takagi et al. had similar results to that of Hartman in regards to mTOR activity. To determine the localization of FNIP1, FNIP2, and FLCN, epitope-tagged proteins were transfected into Cos7 cells. Myc-FNIP2 when expressed alone was distributed within the cytoplasm whereas Flag-FLCN when expressed alone was found in the nucleus. Singular expression of Myc-FNIP1 led to cytoplasmic distribution, whereas co-expression of Myc-FNIP2 and Flag-FLCN resulted in a reticular pattern between the two proteins in the cytoplasm. Co-expression of FNIP1 and Flag-FLCN resulted in localization in the cytoplasm in a fashion analogous to that of FNIP2.

RNAi was used in HeLa cells expressing FLCN, FNIP1, and FNIP 2 to analyze mTOR activity during knockdown experiments. In FLCN suppressed cells, S6K1 was attenuated, indicating a decrease in mTOR activity. FNIP1 suppression caused a significant reduction in S6K1; FNIP2 suppression resulted in a subtle reduction in S6K1 compared to controls. The reduction in S6K1 led to the postulation that FLCN-FNIP1 or FLCN-FNIP2 complexes positively regulate the phosphorylation of S6K1. Treatment with FLCN, FNIP1, or FNIP2 siRNAs led to a decrease in total mTOR level and levels of p-mTOR.

Hudon et al. conducted a study which corroborated the results of the aforementioned studies by observing two categories of renal cysts. One type of renal cysts had increased p-S6 expression, which correlated with large polycystic lesions, whereas the other was negative for p-S6 correlating with smaller single cysts. The increased p-S6 expression correlated with the data of Hasumi et al. and Furuya et al. in which specific deletions of FLCN increased expression of p-S6 and mTORC1 staining in cystic tissue. Hudon et al. as in the case of Hartman observed cysts staining negative for p-S6. Moreover, Hudon et al. noted increased and diminished activation of S6 as a result of increased and diminished expression of FLCN, when human RCC cells were used to grow solid tumors subcutaneously; however when solid tumors were grown in vitro regardless of expression of FLCN and culture conditions, no changes in S6 activation were observed. The activation of S6 in regards to FLCN may be cellular context driven, accounting for the differences noted in the aforementioned studies.

Corroborating the impact of the cellular context driven effects of FLCN, Khabibullin et al. observed discrepancies between Human Bronchial Epithelial Cells (HBE Cells) and Human Small Airway Epithelial Cells (SAECs). Downregulation of FLCN in HBE cells resulted in no significant changes in the levels of phosphorylation of ribosomal protein S6 or sequestreome1/p62. On the other hand, downregulation of FLCN in SAECs resulted in a two-fold increase in phosphorylated ribosomal protein S6 but no change in sequestreome 1/p62.

**Figure 5: The FLCN mediated negative feedback inhibition of the PI3K-AKT-mTOR axis**

**FOLLICULIN AND THE AMPK SIGNAL TRANSDUCTION PATHWAY**

AMP-activated protein kinase (AMPK) is a heterotrimeric Serine/Threonine kinase that functions as a central regulator in maintaining cellular energy homeostasis and energy sensing. AMPK is activated in response to stresses which reduce cellular ATP levels such as low glucose, ischemia, heat shock, and hypoxia. ATP consuming processes cause an increase in AMP/ATP ratio resulting in the activation of AMPK. In response to energy signals indicating low ATP levels, AMPK positively regulates signaling to restore ATP levels, as well as autophagy and fatty acid oxidation. Under these circumstances, AMPK also negatively regulates ATP consuming pathways such as gluconeogenesis, lipid synthesis and protein synthesis.
AMPK dysregulation has been implicated in various cancer types, working with oncogenes to alter tumor cell metabolism and increase cell proliferation. Furthermore AMPK provides cancerous cells a growth advantage in the ability to adapt to metabolic stress. In addition to its role in energy signaling, AMPK is also a central actor in stress driven tumor suppressor network regulation of cell proliferation. The tumor suppressor LKB1 regulates AMPK resulting in the inhibition of mTOR through TSC2. The tumor suppressors TSC2 and p53 are downstream targets of AMPK. AMPK phosphorylates TSC2 to increase its activity. In conjunction with TSC1 the formation of a TSC1/TSC2 functional complex inhibits phosphorylation of S6K (regulator of translation) by mTOR.

The G1/S boundary checkpoint is glucose dependent, AMPK regulated, and will stay in arrest despite active mTOR and amino acid availability should glucose be deprived. AMPK phosphorylates serine 15 on p53, promoting cell survival in response to low glucose levels. Upon restoration of glucose levels the arrested cells will readily reenter the cell cycle. Notably, continuous activation of AMPK results in accelerated p53-dependent cell senescence. Thus, AMPK has the ability to promote or suppress cancer. Clearly, tumorigenesis is driven by cellular context including: the degree and cause of AMPK activation, the expression of AMPK isoforms, and the effects of the other signal transduction pathways within the cell. Losing the LKB1-AMPK signaling pathway may be favorable for tumor cells in the presence of available resources because they may gain increased mTOR activity, and gain the ability to bypass cell growth checkpoints. Conversely, the loss of AMPK also results in a negative impact on tumor growth because AMPK disruption results in disordered energy homeostasis, impaired NADPH/redox regulation, and decreased autophagy (Figure 6). Further, the loss of AMPK prevents cells from taking advantage of cellular metabolic plasticity and adaptation in unfavorable growth conditions. Another factor, eukaryotic elongation factor 2 kinase (eEF2K), is also activated by AMPK and provides cells with the ability to survive in nutrient deprived environments by blocking translation elongation. Tumor cells activate this pathway to adapt to stressful environments. The absence of AMPK will prevent tumor cells from making use of their advantageous growth capability.

Yan et al. in an attempt to understand AMPK induction of the Warburg effect, knocked out FLCN in AMPKα-null, AMPKα-cells using EV control or shRNA, and compared them to AMPKα+/+ cells. The Warburg effect, which is observed in highly proliferative and cancerous cells, is characterized by substantial amounts of pyruvate, produced from glycolysis and redirected from the mitochondria to the lactate dehydrogenase. This results in the high level production of lactate, a process normally reserved for cells in a low oxygen state. Succinctly, the Warburg effect, alternatively known as aerobic glycolysis, is the cellular production of lactate in the presence of oxygen. Knockdown of FLCN resulted in Western blot confirmed increased levels of AMPK and AMPK phosphorylation target PGC-1α in normal oxygen conditions and the absence of energetic stress. FLCN loss in AMPKα−/− was compared to AMPKα−/− cells in an effort to confirm FLCN knockdown was the cause of increased AMPK activation and phosphorylation of PGC-1α. These experiment demonstrated that FLCN downregulation in AMPKα−/− cells resulted in no change in PGC-1α whilst downregulation in AMPK−/−α cells resulted in increased PGC-1α expression. FLCN downregulation in AMPK−/− and AMPKα−/− resulted in increased and unchanged levels of ROS, respectively. The significance of these observations to the Warburg effect is as follows: loss of FLCN results in constitutive activation of AMPK, which results in increased PGC-1α mediated mitochondrial biogenesis and ROS. ROS induces transcriptional activity of HIF and metabolic reprogramming to that of aerobic glycolysis. Moreover, further substantiate that FLCN binding to AMPK prevents activation of the kinase, FLCN expression was rescued in knock out mouse embryonic fibroblasts (KO MEFS) with a Flag-EV, Flag-FLCN-S62A mutant, or Flag-FLCN-WT. The S62A mutant has been shown to have a reduced affinity to AMPKα and FNIP1 and a resultant enhanced phosphorylation of AMPKα. Furthermore, the S62A mutant failed to repress HIF activation.

Further support of Folliculin regulation of AMPK was determined by Possik et al. through FLCN knockdown in Caenorhabditis elegans (C. elegans). The C. elegans FLCN protein has a 50% similarity and 28% identity conservation in comparison to human FLCN. To knockout the FLCN gene a strain of C. elegans carrying flcn-1 (ok975) was utilized. This mutation is an 817 base pair insertion-deletion, which results in a loss of function allele. The flcn-1 (ok975) C. elegans did not exhibit any noticeable developmental differences compared to the wild type. Moreover, the lifespan of the wild type compared well to the knockout C. elegans under non-stress conditions. To induce stress and determine the potential interaction between aak-2 (AMPK ortholog) and FLCN the C. elegans, the superoxide inducer Paraquat was used. The AMPK ortholog, aak-2, increases tolerance to oxidative stress, heat, dietary restriction, anoxia and induces lifespan extension. The truncated FLCN mutation increased resistance to 4mM and 100 mM Paraquat. 

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**Figure 6**: The dichotomous consequences of AMPK activation. AMPK On/Off may result in either pro-tumor or anti-tumor characteristics. 

CURRENT & POTENTIAL THERAPIES

Currently there is no specific treatment for Birt-Hogg-Dubé, and individuals afflicted with the syndrome are treated for the manifestations of the disease. Treatments for fibrofolliculomas include surgical removal, dermabrasion, or electro-desiccation. Unfortunately, with each of the following treatments, there remains a possibility of lesions reoccurring. Renal cancers may be treated with open or laparoscopic partial nephrectomy in order to spare nephrons. For tumors smaller than 3 centimeters, cryoablation or radiofrequency ablation may be considered. The primary objective of any renal neoplasity is to preserve as much of the kidney as possible. Collapsed lung treatment is performed through surgery or aspiration.

The mammalian target of rapamycin (mTOR) activation has been exhibited in FLCN knockdown mammalian cells and is a potential target for BHD. Rapamycin (Generic Name: sirolimus) is produced by Streptomyces hygroscopicus, and was initially isolated for its antifungal properties. Rapamycin has been determined to have anti-tumor and immunosuppressive properties. Initially, rapamycin, a lipophilic macrocyclic lactone, forms a complex with the intracellular binding proteins (FKBPs). The rapamycin:FKBP complex directly binds to mTOR blocking its functions (Figure 7). The inhibition of mTOR prevents cellular progression from G1 to S phase in smooth muscle cells, osteosarcoma cells, and T cells.

Currently, the US National Institute of Cancer has a phase II clinical trial to determine the efficacy of everolimus (rapamycin analog) in BHD patients. Everolimus, which is similar to rapamycin, forms a complex with FKBP to inhibit mTOR. BHD patients are known to have activated mTORC1 and mTORC2. Because of this, Hasumi postulated that both mTORC1 and mTORC2 would have to be inhibited to effectively treat BHD. Rapamycin and its derivatives (Everolimus & Temsirolimus) exert their effects by the FKBP complex on unbound mTOR and mTORC1. It was previously believed that the clinical efficacy of rapamycin and its' analogs were limited to the mTORC1 complex. Sarbassov et al. demonstrated that prolonged rapamycin treatment reduced mTORC2 levels to an extent that disallowed AKT/PKB signaling. The Rapamycin: FKBP complex is unable to bind to mTORC2; however it is capable of binding to newly synthesized, free mTOR molecules. The binding of Rapamycin: FKBP to free mTOR molecules interferes with mTORC2 assembly by preventing the binding of Rictor. To determine the possibility of mTORC2 inhibition, HeLa and human prostate cancer cell lines (PC3) were treated with 100nM of rapamycin at 0.5, 1, 2 and 24 hours and evaluated for Rictor bound to mTOR. The hour of note was 24, at which time in HeLa cells, partial loss of Rictor from mTOR occurred and PC3 cells had almost complete loss of Rictor from mTOR. Treatment of HeLa and PC3 with rapamycin for 48 and 72 hours produced identical results. The mTORC2 complex of mTOR, Rictor, and mLST8 regulates AKT/PKB by phosphorylating S473. Zeng et al. conducted the first study to provide evidence of the ability of rapamycin analogs to inhibit AKT signaling in vitro and in vivo in acute myeloid lymphoma (AML) cells. Treatment of 8 AML samples with temsirolimus in vitro for 24 hours resulted in an 80% decrease of mTOC1 formation and a 50% decrease in mTORC2 formation. To determine clinical relevance to the AML observations, peripheral blood samples obtained from patients with hematologic malignancies treated with temsirolimus (25 mg IV, weekly) and everolimus (5 or 10 mg orally, daily) were analyzed. In 3 of 5 patient samples treated with temsirolimus at 1 or 24 hours (s) and in 6 of 8 patient samples treated with everolimus, levels of phosphorylated Serine 473-AKT decreased.

Dual mTOR/P13K inhibitors are another potential treatment for BHD. The dual inhibitors NVP-BEZ235, GDC-0980, PI-103, PF-04691502, PKI-587, GKS2126458, and XL765/SAR245409 are currently in clinical trials. These dual inhibitors target isoforms of P13K (p110α, β, γ) and the ATP binding sites of both mTORC1 and mTORC2. Serra et al. compared the efficacy of NVP-BEZ235 vs. everolimus in 21 cancer cell lines of varying origins and mutation status. The dual inhibitor NVP-BEZ235 was consistently more effective in the inhibition of cellular proliferation at concentrations over 10 nMolar when compared to everolimus. Both NVP-BEZ235 and everolimus at low dosages inhibit mTOR signaling. However the antitumorigenic effects of everolimus plateau at higher concentrations (> 100 nMolar), whereas NVP-BEZ235 continues to increase its anti-proliferative effects. At these higher concentrations NVP-BEZ235 is capable of inhibiting p110. Engelman et al. demonstrated NVP-BEZ235 lacks efficacy as a single agent in KRAS mutated lung cancers, suggesting that the dual inhibitor may be effective only in particular tissues. The study determined that NVP-BEZ235 was effective in reducing lung adenocarcinomas in tumors maintained by p110α H1047R (catalytic subunit of PI3K) and mutation status. However, after NVP-BEZ235 use in tumors maintained by KRAS tumor reduction was insignificant based on examination with MRI, PET-CT, and microscopic examination. When NVP-BEZ235 was used in combination with mitogen-activated protein kinase inhibitor (MEK), ARRY-142886, there was a reduction in KRAS driven cancers.

![Figure 7](Image)

**Figure 7:** The mechanism of action of TOR inhibition by Rapamycin (SRL). SRL forms a complex with intracellular binding proteins, FKBP. This complex directly binds to TOR to block its function.
References


